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Relative Fecal Abundance of Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Strains and Their Occurrence in Urinary Tract Infections in Women

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Extended-spectrum-beta-lactamase (ESBL)-producing *Escherichia coli* (ESBL *E. coli*) strains are of major concern because few antibiotics remain active against these bacteria. We investigated the association between the fecal relative abundance (RA) of ESBL-producing *E. coli* (ESBL-RA) and the occurrence of ESBL *E. coli* urinary tract infections (UTIs). The first stool samples passed after suspicion of UTI from 310 women with subsequently confirmed *E. coli* UTIs were sampled and tested for ESBL-RA by culture on selective agar. Predictive values of ESBL-RA for ESBL *E. coli* UTI were analyzed for women who were not exposed to antibiotics when the stool was passed. ESBL *E. coli* isolates were characterized for ESBL type, phylogroup, relatedness, and virulence factors. The prevalence of ESBL *E. coli* fecal carriage was 20.3%, with ESBL *E. coli* UTIs being present in 12.3% of the women. The mean ESBL-RA (95% confidence interval [CI]) was 13-fold higher in women exposed to antibiotics at the time of sampling than in those not exposed (14.3% [range, 5.6% to 36.9%] versus 1.1% [range, 0.32% to 3.6%], respectively; $P < 0.001$) and 18-fold higher in women with ESBL *E. coli* UTI than in those with another *E. coli* UTI (10.0% [range, 0.54% to 100%] versus 0.56% [range, 0.15% to 2.1%[, respectively; $P < 0.05$). An ESBL-RA of <0.1% was 100% predictive of a non-ESBL *E. coli* UTI. ESBL type, phylogroup, relatedness, and virulence factors were not found to be associated with ESBL-RA. In conclusion, ESBL-RA was linked to the occurrence of ESBL *E. coli* UTI in women who were not exposed to antibiotics and who had the same clone of *E. coli* in urine samples and fecal samples. Especially, a low ESBL-RA appeared to be associated with a low risk of ESBL *E. coli* infection.

Escherichia coli is a commensal bacterium of the human intestinal tract, with a normal density of colonization (DC) of 10^7 to 10^8 CFU per gram of feces. The *E. coli* intestinal population includes one or more clones, and the relative abundance (RA) of each clone varies (1). Usually, antibiotic-susceptible *E. coli* bacteria form the dominant population, with resistant *E. coli* being subdominant (2). However, the proportion of resistant clones increases with antibiotic exposure (3, 4). *E. coli* is also a major pathogen and is the leading cause of urinary tract infections (UTIs) (5). *E. coli* strains that cause UTIs are commonly accepted to originate from the intestine (6). However, which of the intestinal *E. coli* strains will cause infection remains unclear (5). The prominent *E. coli* clones typically colonize the urethra more often than the subdominant ones (7), but it has been suggested that subdominant *E. coli* strains may overcome their disadvantage for causing UTI when carrying specific virulence factors, such as adhesins or siderophores, which may help the bacteria to survive and multiply in the urinary tract (8). This hypothesis has hardly been confirmed in clinical studies due to the difficulty of quantifying the infecting *E. coli* clones within the total intestinal population when the clone is subdominant. However, this issue can be resolved by focusing on antibiotic-resistant *E. coli* strains, which are easily detectable in feces by using selective agar, even when present in small numbers (9).

Extended-spectrum cephalosporins are often used for the treatment of upper UTIs (10). Resistance to this class of antibiotics has been increasing among *E. coli* strains that cause UTIs, both in hospitals and in the community, as a result of a worldwide dissemination of extended-spectrum-beta-lactamase (ESBL)-producing strains, particularly those of the CTX-M type (11, 12). This increase has been fueled by intestinal colonization, which occurs more frequently than actual infections (4). In our study, we assessed the relationship between the fecal RA of ESBL *E. coli* (ESBL-RA) and the occurrence of ESBL *E. coli* UTIs in women in the community.

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TABLE 1 Characteristics of the populations included in this study

Parameter	Value for country				*P* value
	Moldova	Turkey	Romania	Greece	
Type of center	Primary care	Tertiary hospital	Primary care	Tertiary hospital	
No. of patients	184	76	39	11	
Median age of patients (yr) (range)	31 (17–79)	50 (19–92)	61 (22–81)	49 (30–76)	<0.001
No. (%) of patients with exposure to antibiotics	128 (69.6)	42 (55.3)	8 (20.5)	3 (27.3)	<0.001
No. (%) of pregnant patients	94 (51.1)	6 (7.9)	0 (0)	0 (0)	<0.001
No. (%) of patients with antecedent UTI	160 (87.0)	55 (72.4)	38 (97.4)	10 (90.9)	<0.01
No. (%) of patients with diabetes	15 (8.2)	17 (22.4)	4 (10.3)	0 (0)	<0.01
No. (%) of patients with hospitalization for <3 mo	68 (37.0)	21 (27.6)	7 (17.9)	0 (0)	<0.05

MATERIALS AND METHODS

Patients and strains. We performed a cross-sectional study. Patients were included from 5 centers (Table 1) located in countries with a high prevalence of ESBL *E. coli* (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net), including the National Center for Preventive Medicine, Chişinău, Moldova (May 2009 to June 2010); The Modus Vivendi community-based laboratory, Chişinău, Moldova (February 2009 to September 2010); the Cantacuzino Institute, Bucharest, Romania (October 2008 to July 2009); the Infectious Diseases Polyclinic of the Ege University Teaching Hospital, Izmir, Turkey (September 2010 to March 2011); and the Internal Medicine Polyclinic of the Attikon University Teaching Hospital, Athens, Greece (January 2010 to February 2011). A local investigator from each center was trained at the central laboratory (Bacteriology Laboratory, Bichat-Claude Bernard Hospital, Paris, France) in order to ensure experimental homogeneity among sites.

Female outpatients consulting these centers for UTI symptoms throughout the study period were considered for enrollment after an appropriate urine sample had been taken. They were asked to store the first stool passed after their initial visit at 4°C in a specifically provided container and to bring it back when returning for definitive urine test results. Patients returning after 72 h were excluded from the study. The same standardized questionnaire was used at all sites to record demographic data, antecedent UTI, recent antibiotic use, hospitalization in the last 3 months, pregnancy, and diabetes. All ethical and informed consent rules from each country were followed. Patients diagnosed with UTI caused by *E. coli* upon return were further included in the study.

Approximately 100 mg of each stool sample was diluted in 1 ml of brain heart infusion broth supplemented with 10% glycerol and maintained at −80°C by the local investigators. Stool samples and UTI strains (one per patient) were frozen and kept on dry ice during transport to the central laboratory, where they were kept frozen until use. There, UTI strains were retested for antibiotic susceptibility; ESBL presence was detected by the disc diffusion method, as recommended by the French Society for Microbiology (http://www.sfm-microbiologie.org/). Batches of stool samples were defrosted at 4°C, and 100 μl of each broth was plated onto Drigalski agar plates, with or without 1 mg/liter of cefotaxime, and cultured for 48 h at 37°C. All CFU with distinct morphotypes were further identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany) and tested for antibiotic susceptibility by the disc diffusion method. Densities of total enterobacteria and of ESBL *E. coli* were determined by plating serial dilutions of the broth onto Drigalski agar, with or without 1 mg/liter cefotaxime. CFU were counted in decimal logarithms at the dilution in which 1 to 100 CFU grew. ESBL-RA was calculated as the ratio of the ESBL *E. coli* counts divided by the total number of enterobacteria, expressed as a percentage. For women who carried more than one ESBL *E. coli* clone, the ESBL-RA of the dominant clone was considered.

Antibiotic exposure. No reliable information on previous exposure to antibiotics was available, except for the current episode. The women were categorized as being exposed to antibiotics at the time of fecal sampling if they declared that they had taken antibiotics for the current infection or if antibiotic activity was detected in their fecal samples. Fecal antibiotic activity was detected by using a simple microbiological assay, performed as described previously (13). Briefly, 10 μl of each defrosted stool sample was placed onto antibiotic-free, sterile, 6-mm-diameter paper discs (Dutscher, Brumath, France). The discs were then placed onto Mueller-Hinton agar containing a 10^5-CFU/ml suspension of a fully susceptible *E. coli* strain (ATCC 25922; American Type Culture Collection, Manassas, VA, USA). Stool samples for which a zone of inhibition was observed around the disc, following overnight incubation at 37°C, were determined to have antibacterial activity. Studies in which volunteers were experimentally exposed to antibiotics have shown that this test is always negative for samples taken before drug exposure began (14).

Molecular characterization of ESBL strains. All ESBL *E. coli* strains from urine samples and fecal samples were further characterized. DNA was extracted by using a Triton-based lysis buffer combined with microbeads and further quantified with a NanoDrop quantifier (Thermo Scientific, Waltham, MA, USA). Group 1 and 9 $bla_{\text{CTX-M}}$ genes were amplified and sequenced by using previously described primers (15). If negative, other ESBL-encoding genes (group 2, 8, and 25 CTX-M, TEM, and SHV) were sought (15). Phylogroups were determined by triplex PCR, as described previously (16). Virulence factors harboring the *sfa/foc*, *iroN*, *cnf1*, *hlyC*, *aer*, *fyuA*, *irp2*, *ireA*, *iha*, *ibeA*, *sat*, *neuC*, and *usp* genes were detected by PCR, as described previously (17), and virulence scores were calculated (18).

Concordance between *E. coli* strains from urine and fecal samples in ESBL *E. coli* carriers. The relatedness between ESBL *E. coli* strains from urine and fecal samples was assessed by repetitive element palindromic PCR (rep-PCR) using primers REP1R (5′-NNNGCGCCGNCATCAGGC-3′) and REP2R (5′-ACGTCTTATCAGGCCTAC-3′) (19). Rep-PCR products were migrated on an Agilent 2100 Bioanalyzer with DiversiLab software (bioMérieux, Marcy l'Etoile, France). "Concordant" women were those with urine and fecal samples that contained ESBL *E. coli* strains that had ≥95% similarity. "Discordant" women refers to those for whom the ESBL *E. coli* strains from urine and fecal samples had <95% similarity or for whom there was no ESBL *E. coli* in the urine sample.

Statistical analysis. Data were analyzed by using GraphPad PRISM version 5.04. Qualitative variables were tested by χ^2 or Fisher's exact test. Associations between continuous variables and qualitative variables were tested by Student's *t* tests or by analysis of variance. The significance level was set at a value of 0.05.

RESULTS

ESBL *E. coli* prevalence in urine and fecal samples. In total, 310 women with *E. coli* UTIs returned a stool sample within 72 h after their first visit, including 184 from Moldova, 76 from Turkey, 39 from Romania, and 11 from Greece (Table 1). The median age of the women was 35 years (range, 17 to 92 years). The overall prev-

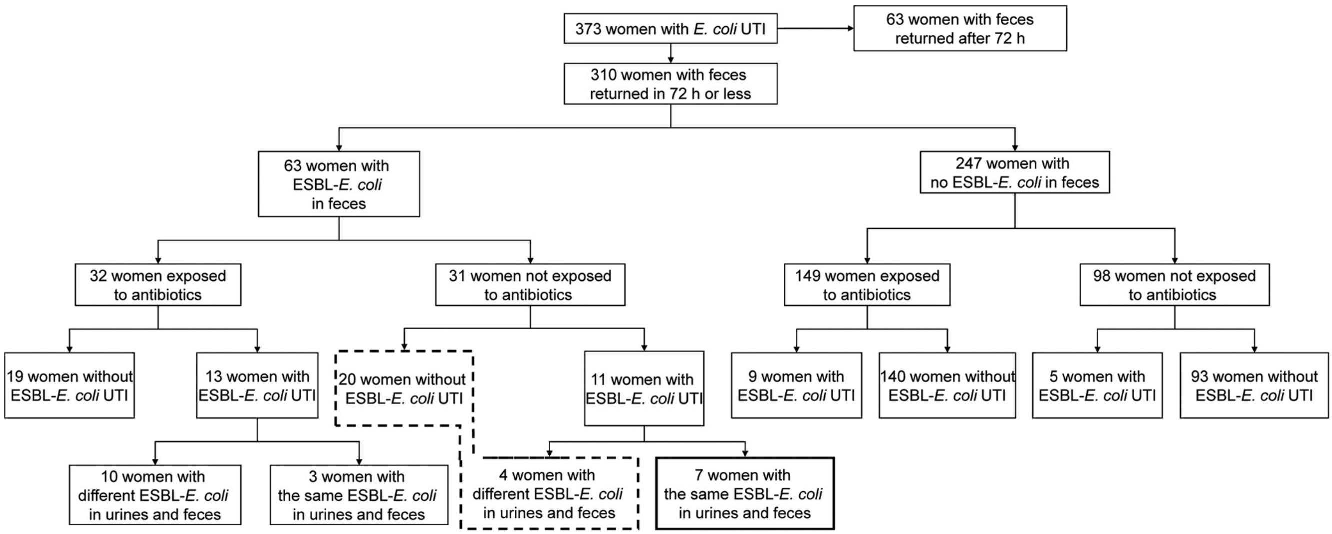

FIG 1 Flow chart of the study. Dashed boxes represent discordant women, and solid boxes represent concordant women (see Materials and Methods).

alence of fecal carriage of ESBL *E. coli* among all sites was 63/310 (20.3%) (Fig. 1). These rates varied by country, with 9.8% in Moldova, 47.4% in Turkey, 17.9% in Romania, and 18.2% in Greece. Among the 63 carriers, 58 (92.1%) and 5 (7.9%) carried 1 and 2 ESBL *E. coli* strains, respectively. The ESBL *E. coli* prevalence in UTI was 12.3% (38/310), with 63.2% (24/38) of women being fecal carriers at the time of sampling. Thus, there were 14 women with an ESBL *E. coli* UTI who were not detected as ESBL fecal carriers. Among them, 1 carried an SHV-2-producing *Klebsiella pneumoniae* isolate, which may have outnumbered an ESBL *E. coli* isolate on the plates; 9 were exposed to antibiotics at the time of sampling; and 2 had $<10^3$ enterobacteria per gram of feces, which may have interfered with bacterial counts. No explanation was found for the remaining 2 women.

CTX-M-15 was the predominant ESBL allele (69.8%), followed by CTX-M-14 (13.2%) and CTX-M-3 (11.3%), without significant differences in type distribution between urine and fecal strains, yet the numbers in the subgroups were low (Table 2). In contrast, ESBL *E. coli* phylogroups were unevenly distributed ($P < 0.05$), with phylogroup A (69.1%) being dominant over phylogroups B2 (17.7%) and D (13.2%) among fecal strains, whereas phylogroups B2, A, and D were evenly represented among the urine strains (Table 2). Among ESBL *E. coli* fecal carriers, 51.6% (32/63) (Fig. 1) were exposed to antibiotics at the time of sampling, either because they declared having taken antibiotics for the current episode of UTI ($n = 30$) or because antibacterial activity was detected in their feces ($n = 2$), without an affirmative declaration.

Associations with ESBL-RA. The mean ESBL-RA (95% confidence interval [CI]) was 13-fold higher in women exposed to antibiotics when the stool was passed than in those not exposed (14.3% [range, 5.6% to 36.9%] versus 1.1% [range, 0.32% to 3.6%], respectively; $P < 0.001$) (Fig. 2). Thus, because antibiotic exposure influenced ESBL-RA, the relationship between ESBL-RA and the occurrence of ESBL *E. coli* UTI was analyzed only among the 31 women (11 with ESBL *E. coli* UTI and 20 with non-ESBL *E. coli* UTI) (Fig. 1) who were not exposed to antibiotics when the stool was passed. ESBL-RA did not vary significantly as a

TABLE 2 Characterization of ESBL *E. coli* from urine and stool samples from the 63 ESBL *E. coli*-carrying women

	No. of samples with ESBL										
	Urine ($n = 38$)					Stool ($n = 63$)[a]					
ESBL	Phylogroup A	Phylogroup B1	Phylogroup B2	Phylogroup D	Total	Phylogroup A	Phylogroup B1	Phylogroup B2	Phylogroup D	Total	Total
Group 1 CTX-M											
CTX-M-15	10	0	11	7	28	32	0	8	6	46	74
CTX-M-1	1	0	0	0	1	4	0	0	0	4	5
CTX-M-3	1	0	0	4	5	4	0	0	3	7	12
Group 9 CTX-M											
CTX-M-14	0	0	3	0	3	7	0	4	0	11	14
CTX-M-16	0	0	0	1	1	0	0	0	0	0	1
Total	12	0	14	12	38	47	0	12	9	68	106

[a] Including 5 women with 2 different ESBL *E. coli* strains producing the respective CTX-M alleles (1 woman each): CTX-M alleles 15 and 1, 15 and 3, 15 and 14, 15 and 15, and 1 and 1.

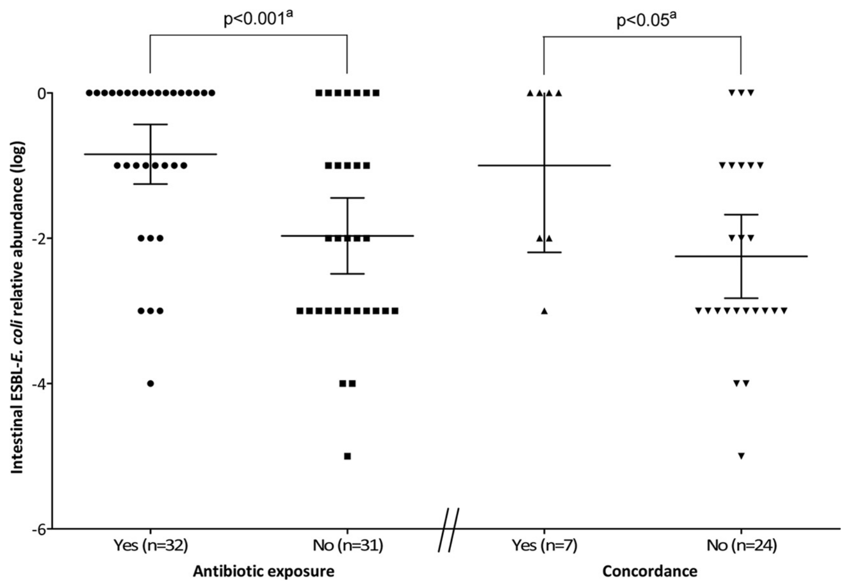

FIG 2 Intestinal ESBL-RA in 63 women according to antibiotic exposure and to the concordance of urinary and fecal ESBL strains (concordance, ≥95% similarity between rep-PCR patterns [see Materials and Methods]) among the 31 women not exposed to antibiotics. The main horizontal bar represents the mean. Error bars represent 95% confidence intervals. [a], determined by a two-tailed, unpaired *t* test.

function of the day on which the stool sample was returned. Stool samples returned on day 0 ($n = 5$), day 1 ($n = 5$), day 2 ($n = 17$), and day 3 ($n = 4$) showed mean ESBL-RAs of 2.5% (range, 0.10% to 65.4%), 1.0% (range, 0.017% to 57.0%), 1.0% (range, 0.16% to 6.2%), and 0.56% (range, 0.0011% to 34.1%), respectively (see Fig. S1 in the supplemental material). There was no significant difference in ESBL-RA between carriers of CTX-M-15 *E. coli* ($n = 19$) or another type of CTX-M ($n = 12$) (mean, 0.79% [range, 0.18% to 3.4%] versus 1.8% [range, 0.17% to 18.5%], respectively) (see Fig. S2 in the supplemental material). The mean ESBL-RA was also not correlated with the virulence factor score of the fecal ESBL strains (0.56% [range, 0.050% to 6.4%], 3.2% [range, 0.51% to 19.5%], and 0.52% [range, 0.036% to 7.5%] for virulence scores of 0 to 1, 2 to 3, and 4 to 7, respectively) (see Fig. S2 in the supplemental material). Likewise, the mean ESBL-RA did not correlate with the phylogroup of the fecal ESBL strains (0.90% [range, 0.20% to 4.1%], 0.56% [range, 0.0023% to 73.0%], and 4.0% [range, 0.15 to 96.4%] for phylogroups A, B2, and D, respectively) (see Fig. S2 in the supplemental material). A total of 24 of the 31 women (77.4%) were discordant, either because they had a non-ESBL *E. coli* UTI ($n = 20$) or because the ESBL *E. coli* isolates from their fecal and urine samples had dissimilar rep-PCR patterns ($n = 4$), whereas 7 (22.6%) were concordant (Fig. 1). The mean ESBL-RA was 18-fold higher for the concordant women than for the discordant ones (10.0% [range, 0.54% to 100%] versus 0.56% [range, 0.15% to 2.1%], respectively; $P < 0.05$) (Fig. 2). In contrast, the mean virulence factor scores for the fecal ESBL *E. coli* strains were not significantly different between concordant and discordant women (1.7 [range, 0.050 to 3.4] versus 2.3 [range, 1.4 to 3.1]) (data not shown).

The sensitivity, specificity, and predictive values of ESBL-RA for the presence of ESBL *E. coli* in urine are shown in Table 3. When the ESBL-RA was <0.1%, the negative predictive value for ESBL *E. coli* UTI was 100%. In contrast, positive predictive values (PPVs) reached a maximum of 57% when the ESBL-RA was ≥10%.

DISCUSSION

Our most important result was that the fecal ESBL-RA was linked to the occurrence of UTI caused by ESBL *E. coli* in women who were not exposed to antibiotics and who had the same clone of *E. coli* in urine samples and fecal samples. In particular, we found that women with a low ESBL-RA (<0.1%) had no risk to develop ESBL *E. coli* UTI. Conversely, the risk increased along with the RA, but the PPV peaked to 57% only. This description of a quantitative link between ESBL-RA and ESBL *E. coli* UTI is novel. Indeed, if the predominant fecal *E. coli* strain had previously been reported to be more likely to cause a UTI (7) or to translocate to the bloodstream (20) than a subdominant one, no precise quantification of the phenomenon was available until now. We initially included 310 women in the study, but we performed the final analysis on a relatively small subset (10%) of them in order to ensure maximal homogeneity and eliminate as many biases as possible. In particular, we excluded from the analysis all women who might have taken antibiotics, even if the reported agent had in theory little or no activity against ESBL-RA, because we judged that the answers to the questionnaire might have been unreliable as to the type of

TABLE 3 ESBL-RA as a predictor of UTI caused by ESBL *E. coli* for the 31 women not exposed to antibiotics

	Concordance determined according to[a]:			
ESBL-RA value (%)	Sens	Spec	PPV	NPV
10–100	0.57	0.77	0.57	0.88
1–10	0.57	0.61	0.33	0.84
0.1–1	0.86	0.45	0.35	0.93
0.01–0.1	1.00	0.10	0.26	1.00
0.001–0.01	1.00	0.03	0.23	1.00

[a] Concordance applied to women with urine and fecal samples that contained ESBL *E. coli* strains that displayed ≥95% similarity (see Materials and Methods). Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

antibiotic taken. In spite of this reduced sample size, our results were statistically significant and conclusive.

Another new result was the 13-fold increase in ESBL-RA in women exposed to antibiotics at the time of sampling. The increase in intestinal density of resistant Gram-negative bacilli during antibiotic administration was previously observed with other antibiotics, such as co-trimoxazole (3). However, to the best of our knowledge, it had not been previously quantified for ESBL strains. Whether a more distant exposure would have done the same, and to which extent, could not be tested here due to a lack of reliable information on previous antibiotics used. Since previous antibiotic treatment is also a known risk factor for the occurrence of UTI with resistant bacteria (21–23), our results suggest that, at least in some cases, ESBL *E. coli* UTI occurs as a 2-step phenomenon, which would include first an increase in fecal ESBL-RA following antibiotic exposure in low-count carriers and then the development of ESBL *E. coli* UTI in individuals with an already high ESBL-RA. If so, maintenance of a low ESBL-RA in the feces might help to minimize the probability of developing an ESBL *E. coli* UTI, even if complete eradication is not obtained. If this hypothesis was proven accurate in a larger and prospective study, attempts to prevent increases in ESBL-RA during antibiotic treatments (24) might prove to be very useful. Conversely, previous antibiotic exposure might have eradicated ESBL *E. coli* carriage in some women for whom an ESBL *E. coli* strain was found in urine samples. Indeed, it has been shown that low concentrations of antibiotics may be sufficient to eliminate fecal bacteria if they are over the MIC of the target strains (25). Also, during treatment with fluoroquinolones, fecal concentrations of antibiotics can reach very high levels that overcome the MIC of even resistant strains (26). In other women, antibiotics might have been taken at a time distant from the time of stool emission, which could explain why they were not detected by our test, despite a residual effect on the microbiota (3, 27). Obviously, further studies on the precise role of antibiotic exposure in ESBL-RA are needed.

We chose RA to measure the level of ESBL *E. coli* fecal carriage instead of bacterial DC because variations in the sizes of samples, transport conditions, and thaw cycles may occur despite standardization. We assumed that variations in these parameters would evenly affect counts of total enterobacteria and of ESBL *E. coli*. RA would thus be less subject to technical variations than DC. Also, because RA is a ratio, it can be determined by using rectal swabs for which the precise quantity of the fecal material is unknown, which is not possible with DC. This may be relevant for clinical research because swabs are easier to obtain, on demand, than fecal samples. Recently, rectal swabs were similarly used to assess the RA of KPC-producing *K. pneumoniae* rather than DC (28).

In our study, virulence factors were not observed to have an influence on the propensity of fecal ESBL *E. coli* to cause UTI, which suggests the role of dominance over virulence in the pathogenesis of UTI in women, at least for ESBL *E. coli* strains. Additionally, correlations were not found between phylogroups and ESBL-RA, suggesting that other traits might determine the level of ESBL *E. coli* within *E. coli* populations.

This study has some limitations, however. We assumed that the densities that we observed in the stool samples reflected the densities at the onset of UTI, even if the feces were passed shortly thereafter. We cannot rule out that the densities of ESBL *E. coli* and total enterobacteria might have changed between these time points. Another limitation was that, in the case of carriage of multiple ESBL *E. coli* strains, only the dominant strain could have been detectable. This might explain why we observed a substantial number of discordant women. Indeed, these limitations may have lowered the PPV, as dominant strains at high ESBL-RA were not found to cause UTI. Also, the populations studied were from countries with very diverse health systems, and the types of centers where the women were recruited differed (Table 1). However, this heterogeneity may also be viewed as a strength, since it decreased the possibility that the results resulted from specific characteristics present at a single center.

In conclusion, the level of ESBL *E. coli* intestinal carriage, as measured by fecal ESBL-RA, was linked to the presence of ESBL *E. coli* in the urine of infected patients. New perspectives for the clinical management of ESBL *E. coli* UTI might emerge from this observation.

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